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Substrate and Inhibitor Specificities of Esterase in *Lucilia illustris* Meigen

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The optimal conditions and substrate specificity of whole body esterase (EST) activity, effects of inhibitors (Eserine, Paraoxon, p-HMB, DDVP, DFP) on the enzyme, and ontogeny of the isozymes were determined in *Lucilia illustris* Meigen.

The optimal temperature was 45°C regardless of kind of reacted substrate, α-naphthyl acetate (α-Na), α-naphthyl butylate (α-Nb), and β-naphthyl acetate (β-Na), but the optimal pH showed some regioselectivity to naphthyl group of the esters; pH 7.0 for β-form, pH 7.5 for α-form. The maximum reaction rate was recorded at about 2.5×10^{-4} M of β-Na and α-Na, but 1.0×10^{-4} M of α-Nb.

Among the five EST inhibitors tested, DDVP was the most powerful. However, distinction of the relative specificity of inhibitors between three body parts, head, thorax, and abdomen, was shown, representing differences in the distribution and activity of isozymes. Of 12 carboxyl-esterases (CE), 8 cholinesterases (ChE) and 2 arylesterases (ArE) identified based on their inhibitor specificity throughout the development, two larval and prepupal stage specific ChEs, no pupal specific, and 2 CEs, 2ChEs, and one ArE adult specific isozymes were confirmed.

KEY WORDS: *Lucilia illustris*, Esterase, Inhibitor, Substrate, Optimal Condition

In insects, esterases (EST) act extensively on various kinds of substrate (Sudderudin and Tan, 1973); Turunen and Chippendale, 1976), and show high polymorphism and genetic variation (Korochkin *et al.*, 1973; Mattiensen *et al.*, 1993). The enzymes, although the precies functions and substrate specificities of their isozymes are not clear yet, are known to participate in the regulation of juvenile hormone titer (Whitmore *et al.*, 1972; Newitt and Hammock, 1980), the post-embryonic differentiation (Vedbrat and Whitt, 1974; Kapin and Ahmad, 1980; Ugaki *et al.*, 1983), and many different processes including metabolism of lipids and insecticides (Sudderudin and Tan, 1973; Zhu and Brindley, 1990).

Based on their substrate and inhibitor specificities, esterases are generally classified into three groups; carboxylesterase (CE), arylesterase (ArE), and cholinesterase (ChE)(Mounter and Wittaker, 1953; Vedbrate and Whitt, 1974; Hideaki and Ozaki, 1983). CE implies the isozymes which act vigorously on various simple esters of low molecular, but slightly on phenolicesters (Clements, 1967), and has a important role in the synthesis and transport of cuticular wax (Sudderuddin and Tan, 1973; Ahmad and Forgash, 1976), the regulation of juvenile hormone titer (Sudderuddin, 1973; Sparks *et al.*, 1979), and the detoxification of substances introduced from external environment as insecticides (Khun and Hegmann, 1982; Villani *et al.*, 1983; Fonnum *et al.*, 1985; Oppenoorth, 1985; Zhu and Brindley, 1990; Prabhakaran and Kamble, 1995). ArE is a small group of isozymes

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which function simply in the degardation of aromatic esters including phenolicesters (Clements, 1967; Sudderuddin and Tan, 1973). And ChE is an important regulatory enzyme responsible for controlling the neural transmission on synapses where it acts to hydrolyze the excitatory neurotransmitter, acetylcholine (Silver, 1974; Xu and Bull, 1994). Also ChE is the target of organophosphorus (OP) and carbamate insecticides as it can be readily inhibited by these toxic compounds (Randall and William, 1986; Xu and Bull, 1994).

In the previous report, we described the activity of haemolymph esterase reacted with some inhibitors and its isozymatic composition during development (Yoo *et al.*, 1993). This study was undertaken to confirm the optimal pH and temperature and substrate specificity of whole body esterase, effects of several inhibitors on the enzyme action, and ontogeny of the isozymes during mature larval-pupal-adult development of *Lucilus illustris*.

Materials and Methods

Insect and homogenization

Eggs of *Lucilia illustris* Meigen were trapped in the vicinity of Taejon, and the larvae were reared on fish paste and cattle liver at 25°C, 16h:8h photoperiod and 60% RH. For experiments, whole body was collected at the following stages, respectively; mature larva, prepupa, 1-, 2-, 3-, 4-, and 5-days old pupa, and 1-, 3-, 5-, and 7-days old adult. In particular, adult files were cut into three pieces, head, thorax, and abdomen, and then collected for the analysis of isozyme distribution.

Whole body and the body pieces were homogenized with 67 mM phosphate buffer (pH 7.0) in the ice-chilled glass homogenizer. The extract was centrifuged at 12,000 g for 20min at 4°C. The supernatant obtained was used as the crude enzyme extract.

Enzyme assay

Esterase activity was measured according to Ozaki and Koike (1965). The reaction was started by the adding of substrate solution (5 ml) to 1 ml of sample supernatant at 37°C and pH 7.0 for 20 min. The color was developed by the adding of 1 ml of naphthol diazo blue B to the reactant. And this colored reactant was mixed with 10 ml of ethyl acetate and 1 ml of 40% trichloroacetic acid (TCA), and then centrifugated (6,000 g) for 5 min. The O.D. value of the supernatant was read at 540 nm using spectrophotometer. Unit of esterase activity represents μg of β-naphthol liberated by enzyme action per g of insect body weight. All values in enzyme activity were calulated from three replicate assays with each 20 flies.

Electrophoresis and detection of isozymes

Fifteen μl of the extract was electrophoresed on 7.5% polyacrylamide gel (Davis, 1964) using 0.18M Tris-glycine buffer (pH 8.3) for 1.5hrs at 4 °C, 3 mA constant current per gel, 5mm i.d. and 6.5 cm long. After electrophoresis, the gels were immersed in 7 ml 67 mM phosphate buffer (pH 7.0) containing 4 mg β-naphthol acetate and 7 mg Fast blue RR salt at 25°C for 30 min, then incubated in ethanol:10% acetic acid (3:2, V/V) at 37°C for 30 min. The developed color of the isozyme bands was assessed by densitometry at 540 nm using Beckman Paragon densitometer CDS-200.

Classification of isozymes

The effects of inhibitors on each isozyme were determined by each addition of 1×10^{-5}M eserine sulfate (Eserine), o-o-dimethyl o-(4-nitrophenyl) phosphate (Paraoxon), para-hydroxyl mercuribenzoate (p-HMB), o,o-dimethyl o-(2,2-dichlorovinyl) phosphate (DDVP), and diisopropyl phosphofluoridate (DFP) into the reaction mixture used for the detection of isozymes.

According to Mounter and Whittaker (1953), the isozymes inhibited by both carbamate (Eserine) and organophosphates (Paraoxon, DDVP, DFP) as ChE, the isozymes inhibited by only organophosphates as CE, and the isozymes inhibited by both organophosphates and sulfhydryl reagent (p-HMB) as ArE were classified, respectively.

Optimum pH and temperature, and substrate specificity

The whole body extract collected from 1-day old pupa was used as the enzyme preparation. The effects of pH and temperature and substrate concentration on esterase activity were studied by using three kinds of substrate (α-Na, α-naphthyl acetate; α-Nb, α-naphthyl butylate; β-Na, β-naphthyl acetate), respectively. The enzyme activity was expressed as micro-moles of naphthol produced by 1 mg protein.

Protein contents of enzyme preparation were determined by the method of Lowry *et al.* (1951) with some slight modifications.

Results and Discussion

Table 1 shows the activities of whole body esterase (EST) reated with several kinds of inhibitors during the mature larval-pupal-adult development of *Lucilia illustris*. The activity of non-treated control enzyme decreased during larval-pupal transformation, increased until to 2-days old pupa, decreased again at 4-days old pupa, then somewhat recovered during the late pupal period. The highest value of EST activity was recorded at 1-day old adult in both sexes. It is known that esterase activity displays a cyclic pattern correlated with lipid metabolism (Nowock *et al.*, 1975; Ahmad and Forgash, 1976), feeding activity and development (Kapin and Ahmad, 1980), and reproduction (Sheehan *et al.*, 1979; Yoo *et al.*, 1993). In *Lucilia illustris*, consequently, the high value of EST activity at the early pupal and early adult stage seems to represent the increased lipid metabolitic activity related to histolysis of larval tissues and development of sex organs, respectively.

Five kinds of inhibitor (Eserine, Paraoxon, p-HMB, DDVP, DFP) were used *in vitro* to determine inhibitor specificity of EST. DDVP and p-HMB were very effective inhibitors, but p-HMB was the weakest to head esterase action (Table 2). On the other hand, thorax and abdomen esterase activity were inhibited effectively by DDVP and DFP (Table 3, 4). This distinctive relative specificity of the inhibitors to the body portions was thought to be due to the differences in distribution or activity of EST isozymes (Table 1,

Table 1. Whole body esterase activity and inhibition rate of inhibitors to the enzyme action in *Lucilia illustris* Meigen

Developmental Stages		Esterase Activity*	% Inhibition** of inhibitors to enzyme action				
		Control	Eserine	Paraoxon	p-HMB	DDVP	DFP
Mature larva		883	17.10	42.81	68.52	93.09	56.17
Prepupa		867	11.04	24.91	51.10	92.73	40.14
	1-day old	808	18.54	32.05	60.15	88.00	32.05
	2-days old	916	11.09	31.33	41.70	90.61	53.71
Pupa	3-days old	875	32.34	37.26	59.09	91.43	57.37
	4-days old	807	19.05	47.46	55.64	91.82	41.14
	5-days old	845	15.15	46.39	45.44	92.31	47.22
	1-day old female	998	22.34	42.89	47.19	93.49	44.99
	1-day old male	974	12.83	45.17	36.14	93.53	42.20
	3-days old female	886	18.28	59.59	46.39	93.00	57.11
Adult	3-days old male	944	20.97	58.16	43.43	93.43	51.17
	5-days old female	820	14.51	48.29	57.20	88.41	53.66
	5-days old male	893	18.03	30.35	59.91	88.91	59.01
	7-days old female	856	13.97	55.61	36.24	89.49	27.34
	7-days old male	781	13.80	20.87	28.05	90.01	44.20

*, μg of β-naphthol liberated by enzyme action/g of wet weight

**, % inhibition=$(1-\frac{\text{activity inhibited}}{\text{activity of control}})\times 100$

Table 2. Inhibition rate of inhibitors to head esterase action in *Lucilia illustris* Meigen

Developmental Stages	% Inhibition Rate**				
	Eserine	Paraoxon	p-HMB	DDVP	DFP
1-day old female	70.83	64.44	22.64	93.06	66.81
1-day old male	64.81	73.82	14.45	95.14	71.39
3-days old female	56.75	44.79	51.97	94.02	39.83
3-days old male	55.52	58.44	43.34	94.32	57.31
5-days old female	45.18	35.16	12.29	91.87	48.02
5-days old male	43.10	55.69	32.93	91.04	68.28
7-days old female	66.60	66.03	10.88	93.51	75.19
7-days old male	64.02	67.34	64.02	90.96	79.70

Table 3. Inhibition rate of inhibitors to thorax esterase action in *Lucilia illustris* Meigen

Developmental Stages	% Inhibition Rate**				
	Eserine	Paraoxon	p-HMB	DDVP	DFP
1-day old female	58.80	39.48	21.46	93.71	81.55
1-day old male	53.93	35.43	13.09	94.59	64.05
3-days old female	38.01	40.65	15.24	93.29	76.02
3-days old male	60.37	51.85	18.89	93.52	69.63
5-days old female	55.25	48.43	26.70	93.74	71.27
5-days old male	58.75	49.17	32.97	93.00	70.90
7-days old female	50.21	37.84	15.35	85.89	68.67
7-days old male	59.21	40.79	19.49	89.44	63.98

Table 4. Inhibition rate of inhibitors to abdomen esterase action in *Lucilia illustris* Meigen

Developmental Stages	% Inhibition Rate**				
	Eserine	Paraoxon	p-HMB	DDVP	DFP
1-day old female	65.17	52.10	35.12	94.78	59.07
1-day old male	46.63	30.05	24.96	94.42	47.78
3-days old female	60.99	60.61	23.82	93.12	76.48
3-days old male	62.12	62.48	15.04	93.63	78.23
5-days old female	46.06	56.97	21.68	90.63	61.30
5-days old male	73.31	71.50	52.28	94.63	80.62
7-days old female	50.87	35.49	58.39	89.86	78.50
7-days old male	55.21	49.81	19.11	88.04	68.92

2, 3, 4). In *Lucilia illustris*, the mean of inhibition rate of DDVP to EST was the highest corresponding to the result in *Lymantria* dispar (Kapin and Ahmad, 1980). This phenomenon is due to that CE is the most abundant among the classified isozyme groups throughout the development.

Effects of temperature on the hydrolysis of three kinds of substrate (α-Na, α–Nb, β-Na) were examined from 20 to 55°C. A typical curve for whole body esterase activity-temperature relationship is shown in Fig. 1. Enzyme activity

increased gradually from the lowest at 20°C to the highest at 45°C. Although the response of EST to the change of temperature was different towards different substrates, the basic response patterns were similar. This is in agreement with optimal temperature range (40~45°C) of esterases in other insect species, *Lygus hesperus* (Zhu and Brindley, 1990) and *Aphis gossypii* (Owusu *et al.*, 1994).

The EST action on each substrate, α-Na, α-Nb, or β-Na, was counted from 5.5 to 9.0, at 37°C and substrate conc. 0.3 mM (Fig. 2). The optimal pH showed some regioselectivity to naphthyl group of the substrate esters. When β-Na was reacted as substrate, the activity increased gradually from 5.5 to 7.0, but decreased when pH was above 7.5. However, when α-Na and α-Nb were used, the enzyme activity was showed the maximum at pH 7.5. Working on esterases of larval *Lymantria dispar*, Kapin and Ahmed (1980) reported an optimum pH range of 7.5 to 7.7.

Effects of substrate concentration on the EST activity were examined by measuring the activity at different concentrations of α-Na, α-Nb, and β-Na substrates from 5.0×10^{-6} to 1.0×10^{-3}M (Fig. 3). All examinations were carried out at 37°C and pH 7.0. The maximum reaction rate was recorded at about 2.5×10^{-4}M for β-Na and α-Na, and 1.0

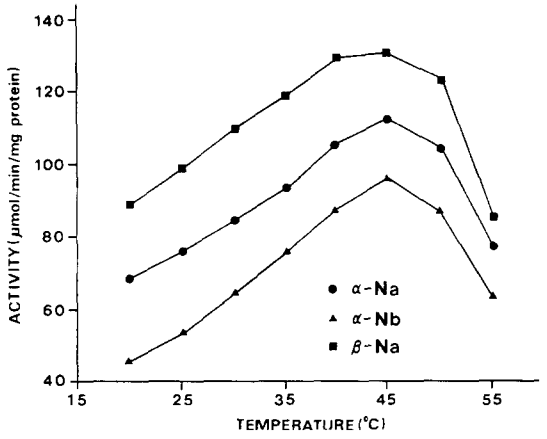

Fig. 1. Effects of temperature on the activity of whole body esterase acting to three kinds of substrate (0.3 mM) at pH 7.0. α-Na, α-naphthyl acetate; α-Nb, α-naphthyl butylate; β-Na, β-naphthyl acetate

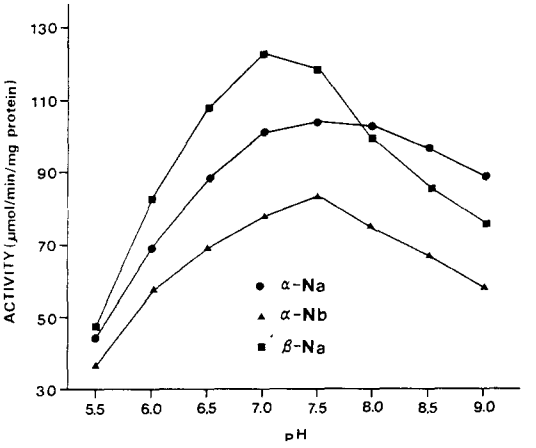

Fig. 2. Effects of pH on the activity of whole body esterase acting to the three kinds of substrate (0.3 mM) at 37°C. The abbreviations are same as in Fig. 2.

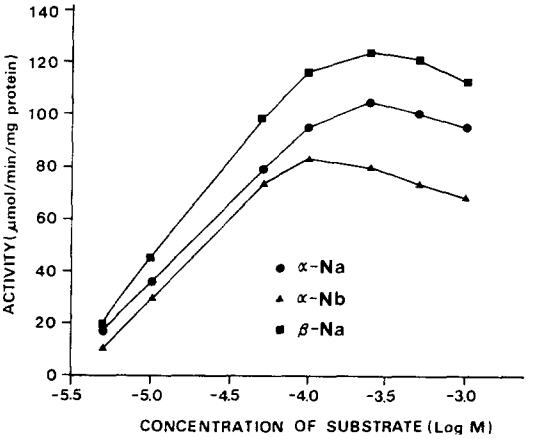

Fig. 3. Effects of substrate concentration on the whole body esterase activity at 37°C, pH 7.0. The abbreviations are same as in Fig. 2.

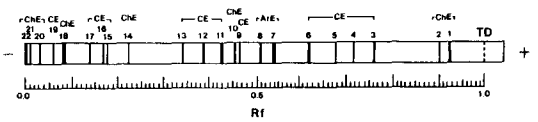

Fig. 4. Zymogram of whole body esterase isozymes detected throughout the development of *Lucilia illustris* Meigen using of 7.5% polyacrylamide gel.

Table 5. Ontogeny of whole body esterase isozymes during development of *Lucilia illustris* Meigen

No. of Bands	Description	Developmental Stages														
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	ChE	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
2	ChE	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
3	CE	+	+	+	+	+	+	−	−	−	+	−	−	−	−	−
4	CE	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
5	CE	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
6	CE	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
7	ArE	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+
8	ArE	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
9	CE	+	−	−	−	−	−	−	+	−	+	−	+	−	−	−
10	ChE	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	CE	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
12	CE	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	CE	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	ChE	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
15	CE	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−
16	CE	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
17	CE	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
18	ChE	−	−	−	+	+	−	+	+	+	+	+	+	+	−	−
19	CE	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
20	ChE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
21	ChE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	ChE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

A, mature larva; B, prepupa; C, 1-day old pupa; D, 2-days old pupa; E, 3-days old pupa; F, 4-days old pupa; G, 5-days old pupa; H, 1-day old female adult; I, 1-day old male adult; J, 3-days old female adult; K, 3-days old male adult; L, 5-days old female adult; M, 5-days old male adult; N, 7-days old female adult; O, 7-days old male adult (+, presence; −, absence).

$\times 10^{-4}$M for α-Nb. These values obtained in *Lucilia illustris* are lower than 3.15×10^{-4}M of non-specific esterase reported by Sudderuddin (1973), as well as 5×10^{-2}M of carboxylesterase reported by Owusu *et al.* (1994). The order of the efficiency for EST hydrolyzing the three substrates was β-Na>α-Na>α-Nb (Figs. 1, 2, 3).

A total of 22 whole body EST isozymes was identified throughout the development of *Lucilia illustris* (Fig. 4), and their ontogeny is shown in Table 5. The detected isozymes were mainly consisted of ChE and CE. Around middle line of the gel, only two ArE bands (Rf 0.50~0.55) were confirmed at mature larval and adult stages.

There was no pupal specific isozyme, but 5 adult specific (bands 2, 8, 11, 14, 19), one larval (band 10) and one prepupal (band 1) specific isozymes were confirmed. All the larval and prepupal isozymes (bands 1, 10) were ChE, whereas adult specific bands were consisted of 2 ChE (bands 2, 14), 2 CEs (bands 11, 19), and one ArE (band 8). On the other hand, three adult female specific isozymes, one ChE (band 2) and 2 CEs (bands 3, 9), were identified, but any male specific bands were not observed.

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연두금파리 Esterase의 기질 및 억제제 특이성에 관한 연구

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연두금파리(*Lucilia illustris* Meigen)에서 충체 esterase(EST) 활성도의 최적조건과 기질 특이성, 억제제들(Eserine, Paraoxon, p-HMB, DDVP, DFP)의 처리효과 및 발생에 따른 isozyme 조성상의 변화를 연구하였다. 최적 온도는 기질로써 α-naphthyl acetate(α-Na), α-naphthyl butylate(α-Nb), 그리고 β-naphthyl acetate(β-Na)들을 반응시켰을 때 모두 45°C로 나타났으나, 최적 pH는 β-form에서 pH 7.0, α-form에서 pH 7.5로써 ester의 naphthyl group에 대한 regioselectivity를 보여주었다. 최대반응 속도는 기질로써 β-naphthyl acetate와 α-naphthyl acetate와의 반응시 약 2.5×10^{-4}M에서 나타났으나 α-naphthyl butylate에서는 약 1.0×10^{-4}M로 나타났다. 다섯 가지 EST 억제제들 가운데 DDVP가 가장 강력한 억제효과를 나타내었다. esterase에 대한 억제제들의 relative specificity가 몸의 부위(두부, 흉부, 복부) 별로 다르게 나타나, isozyme들의 활성 및 분포에 차이가 있음이 시사되었다. 전 발생 단계를 통하여 억제제 특이성을 기준으로 총 12개의 carboxylesterase(CE), 8개의 cholinesterase(ChE) 및 2개의 arylesterase(ArE) band들이 분류, 확인되었는데, 2개의 ChE가 유충 및 전용기에 특이성 있게 나타났으며 2개의 CE, 2개의 ChE 및 1개의 ArE가 성충기에 특이성 있게 출현하였으나 용기 특이성 isozyme은 관찰되지 않았다.